The enzyme DXS as an anti-infective target

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Chapter 3

Development of peptidic inhibitors of the anti-infective target DXS by phage display

In this chapter, we will describe how we discovered the first peptidic inhibitors of DXS by using phage display. An IC_{50} *value of 9.5 ± 2* μM *against* Deinococcus radiodurans *DXS was achieved and an Ala scan revealed essential features of the amino acid sequence (*i.e., *a Ser-Ser-Ser motif) responsible for its inhibitory activity that can be optimized in future design cycles.*

Marcozzi, A.; Masini, T.; Zhu, D.; Pesce, D.; Illarionov, B.; Fischer, M.; Herrmann, A.; Hirsch, A. K. H. Development of potent peptidic inhibitors of the anti-infective target DXS by phage display, *manuscript under review*

3.1 Peptides as therapeutic agents

For many years, peptides have been considered to be poor drug candidates and still nowadays they partially suffer from a "deficit in image".[1] The main limitations attributed to the development of peptides as therapeutic agents involve low oral bioavailability, short half-life caused by their rapid degradation by proteolytic enzymes, rapid clearance (particularly in the liver and the kidneys) and poor ability to cross physiological barriers because of their remarkable hydrophilic character.[2,3] Moreover, the costs for large-scale production of peptides are still higher than for small molecules, although considering the cost of the total drug-development process, the differences are not so significant, given that peptides generally have a higher clinical success rate.[4,5] Furthermore, standard peptide sequences composed of natural amino acids (aa) are, besides few exceptions, worse drug candidates than small organic molecules, given their intrinsic physicochemical properties and pharmacokinetic profiles.

The development and optimization of peptides as therapeutic agents has been hampered by difficulties in their synthesis, routes of administration and quantitative detection once administered but thanks to advances in chemistry, biochemistry, chemical biology and drug formulation and delivery, most of these limits have been overcome. As a result, over the past decade or so, the use of peptides as drug candidates is being increasingly encouraged and pursued, with some of them having reached the market (Table 1).

Table 1. Three examples of marketed peptide-based drugs.

Exanatide (Byetta®)	**Enfuvirtide (Fuzeon®)**	**Buserelin acetate (Bigonist®)**
Amylin Pharms, Eli Lilly	Roche	Sanofi-Aventis
39 aa, antidiabetic agent	36 *aa*, anti-HIV	9 *aa*, advanced prostate cancer

As can be seen in Table 1, the size of a peptide-based drug can vary a lot. On the one hand, efficient target recognition can, in fact, occur also with very few residues. On the other hand, peptides that are 30–40 residues long can enable the binding of very wide binding grooves.

Peptides certainly have several advantages over small, organic molecules, for example the risk of systemic toxicity is reduced, given that their degradation products are amino acids. Moreover, thanks to their short half-life, few peptides accumulate in tissues, with a

reduced risk of complications caused by their metabolites.[1] One of the main advantages of the use of peptides rather than small, organic molecules is that they can be tuned so as to reach extremely high selectivities toward their targets, explaining their elevated success rates in clinical trials.[6]

Thanks to the availability of a wide range of synthetic protocols, both amide bonds and side chains of the amino acid sequences can be modified so as to develop peptides resistant to proteolytic degradation.[7] More complex modifications might involve, for example, the insertion of a knotted Cys core, which endows the peptide with an exceptional thermal and proteolytic stability.[8] Several strategies for improving the drug penetration through biological barriers have been developed, which include the incorporation of positively charged amino acids at the terminal positions (although polycations might destroy mammalian membranes)[9] or the conjugation of the peptide to a ligand of cell-surface receptors, such as a carbohydrate receptor, which results in the incorporation of sugar moieties in the amino acid sequence.[10]

A lot of scientific effort has been put into improving the bioavailability of peptide therapeutics, which has been (and is) one of the main topics of discussion among drug developers and pharmaceutical companies. However, as Otvos and Wade remark, “peptide drugs do not necessarily need to be orally available”: many peptide-based drugs such as insulin or amylin are currently available in patient-friendly packaging ready for subcutaneous self-administration.[4] Many peptides can be efficiently formulated for intranasal administration as well.[11]

3.2 Phage display as a powerful tool for the identification of potential peptide-based drugs.

Phage display is a biological technique introduced by Smith in 1985[12] and the phage-display process is depicted in Figure 1. First, a population of filamentous bacteriophages (commonly referred to as phages, which are viruses that can replicate within bacteria) is engineered with different genes encoding different fusion proteins (Figure 1, step a). To do so, randomized cDNA sequences are inserted into the genome of the phage, specifically into a gene encoding for the phage’s coat protein, causing the phage to express the peptide fused with one of the phage’s coat proteins on its surface. The most important coat proteins for the display of peptides on the surface of phages are pIII (minor coat protein) and pVIII (major coat protein), the former being more widely used, given the

higher avidity effect of pVIII, which leads to the selection of peptides with lower affinities (K_d = 10–100 μM) than the ones selected using pIII proteins (K_d = 1–10 μM). When no information is available about key features to be exploited for the target–peptide recognition process (*e.g.*, key amino acid motifs), randomized libraries are generated, ranging from six to 30 residues. The optimal length required for a randomized library is difficult to establish and predict given that it depends on many variables but it is a key for the success of the phage-display process. As a result, as much information as possible about the protein target, the specific pocket and other known inhibitors of the target should be collected before starting a phage-display project.

Once a library of phages – displaying different peptides – has been created, the library is exposed to the target (Figure 1, step b), those phages that bind the desired target are selected, while the non-binders are washed away (Figure 1, step c).[13] Once the non-binders have been removed, the other phages will be eluted (Figure 1, step d) and amplified in bacterial cells (Figure 1, step e). This cycle is repeated several times so as to amplify the library of the selected phages, helping to select binders with stronger affinity and to reduce the number of non-specific binders. Finally, the specifically bound phages are eluted and isolated. The primary structure of the displayed peptides is identified through DNA sequencing.[14]

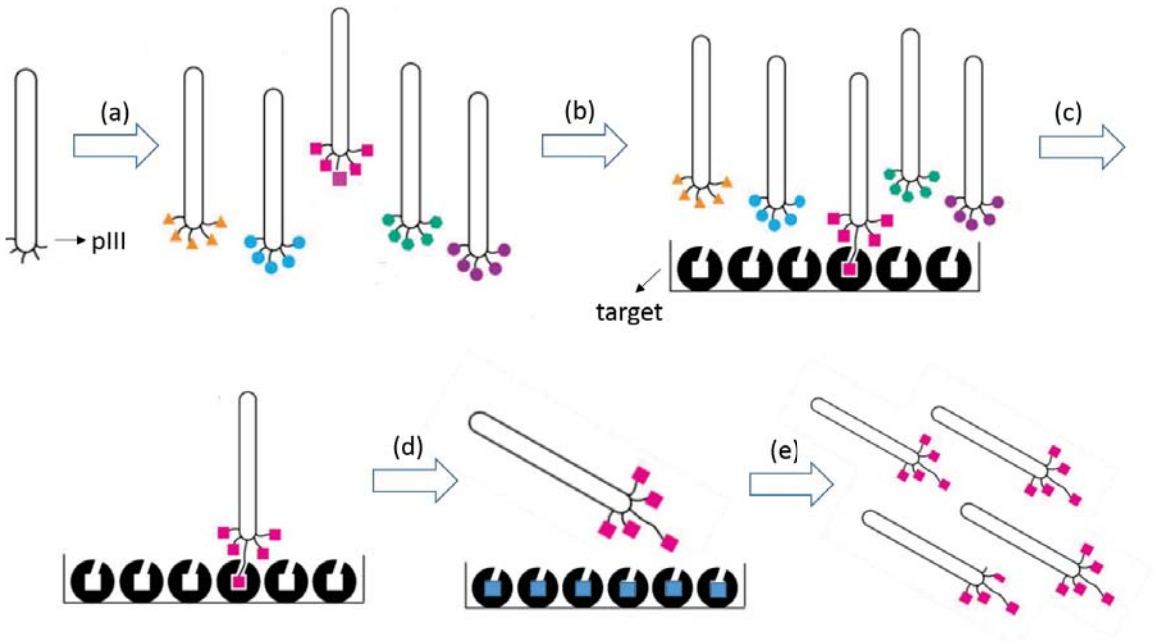

Figure 1. Schematic representation of the phage-display technique.

Phage display has been used as a powerful tool in drug discovery[15] but also in nanostructured electronics,[16] agriculture[17] and neurobiology.[18] In drug discovery, its success is due to its advantages over traditional random screening methods: an enormous variety of peptides is generated and screened against the target, without the need to synthesize and purify them individually, making phage display very cheap, simple and effective, given that it relies on established biochemical methods. Several peptides, discovered by phage display, have been marketed as drugs or are currently in clinical trials.[19]

It should be noted that phage display might lead to the identification of binders of a specific target, which do not necessarily translate into inhibitors. The inhibitory potency of the selected peptide-binders should therefore be tested *in vitro* against the target at a later stage. Moreover, phage display delivers hits, which do not have drug-like properties of a lead compound yet, but have to be further optimized in terms of their potency and especially in terms of their pharmacokinetic properties, as discussed in Section 3.1. For example, the sequence of peginesatide (hematide®), currently in Phase III clinical trials for the treatment of anemia associated with chronic kidney diseases, was originally discovered by phage display[20] but had to be further optimized so as to enter the next stage of the drug-discovery process: PEGylation of its amino acid chain improved several properties such as solubility, stability, increased resistance to proteolysis and increased plasma half-life.[21,22]

3.3 Exploiting phage display as a tool for the identification of peptidic inhibitors of DXS

All inhibitors of DXS reported in the literature to date are small, organic molecules and, to our knowledge, there is no report on peptidic inhibitors of this enzyme. Therefore, we decided to use phage display for the identification of peptidic hits. For the phage-display protocol, we used the model enzyme *D. radiodurans* DXS, given that it is more stable than *M. tuberculosis* DXS. In fact, in a preliminary study, we checked the stability of *D. radiodurans* DXS both at 4 °C and at room temperature by monitoring its activity for up to 37 hours using a coupled enzyme activity assay. The assay employs IspC as the auxiliary enzyme, to enable spectrophotometrical monitoring of the consumption of NADPH. We observed no loss in activity even after 37 hours at room temperature. From these initial tests we concluded that *D. radiodurans* DXS is sufficiently stable to act as a target during the phage-display process.

3.4 The phage-display protocol

During the first step, we screened a fully random M13 bacteriophage peptide library to specifically detect sequences, which are able to bind any part of the surface of *D. radiodurans* DXS. The first phage-display selection protocol was carried out using the commercially available M13 library PhD12 (NEB E8111L), consisting of M13 phages expressing a 12aa peptide at the N-terminus of each coat protein pIII. A small linker Gly-Gly-Gly-Ser was inserted between the peptide and the coat protein to increase the conformational freedom of the exposed peptides and to minimize the contribution of the protein pIII to the overall binding event. The schematic sequence of this library is N-term-X12-GGGS-pIII-C-term. We chose to start by screening libraries containing 12aa peptides because this constitutes a good starting point and a compromise between having too long peptides – which could get stuck by binding at the surface of the protein – and too short peptides – which would not be able to penetrate into the pocket we aim to target. Moreover, given that for *E. coli* cells – the most common hosts used for bacterial surface display – the transformation efficiency is typically 10^9, it does not make sense to start exploring libraries of longer peptides, such as 20aa, which would give rise to very high theoretical complexities (20^{20} possible sequences in the case of a 20aa library). In fact, only a very small fraction of the theoretical library would be explored in this case.

We designed a phage-display protocol where the phages are incubated in solution with *D. radiodurans* DXS. After incubation, magnetic beads are added to allow for the recovery of the target protein-phage complex. In principle, one could add the magnetic beads right from the beginning of the protocol and incubate a solution containing the protein, the phages and the beads. Nevertheless, the risk of selecting phages, which bind to the beads rather than to the target protein, is much higher in this case, due to the prolonged contact time of the beads and the phages during incubation. In order to reduce this risk, we decided to not use this protocol. Moreover, we used a two-step selection approach to identify peptide binders (Table 1): we used two types of magnetic beads and several elution buffers to avoid background-selection bias (Figure 2). Analysis of the selected peptides enabled us to design a new and more stringent library for the second selection step in which we screened for sequences that specifically bind to the cofactor-binding site of *D. radiodurans* DXS, namely the thiamine diphosphate (TDP)-binding site. We used a solution of TDP as competitive eluent to select those phages that interact at the TDP-binding site.

Table 2. Overview of the phage-display protocols used for the first and second selection.

	Phage Display I	**Phage display II**
Library	X12GGGS	XSSX9GGGS
Competitors	None	Wild-type M13
	Rounds I and II	
Target	Desthiobiotin-DXS	His-tag-DXS
Solid support	Streptavidin-coated beads	Nickel-coated beads
Eluent	Biotin	TDP
	Round III	
Target	His-tag-DXS	*Not performed*
Solid support	Nickel-coated beads	*Not performed*
Eluent	Imidazole	*Not performed*

Given that unspecific binders are often present after the second round of selection, we added wild-type M13 phages as competitors: wild-type phages efficiently compete with virus particles expressing the peptide library for unspecific phage–target interactions and they can be easily filtered out during post-sequencing analysis. Both measures decrease the probability of selecting unspecific binders or false positives.

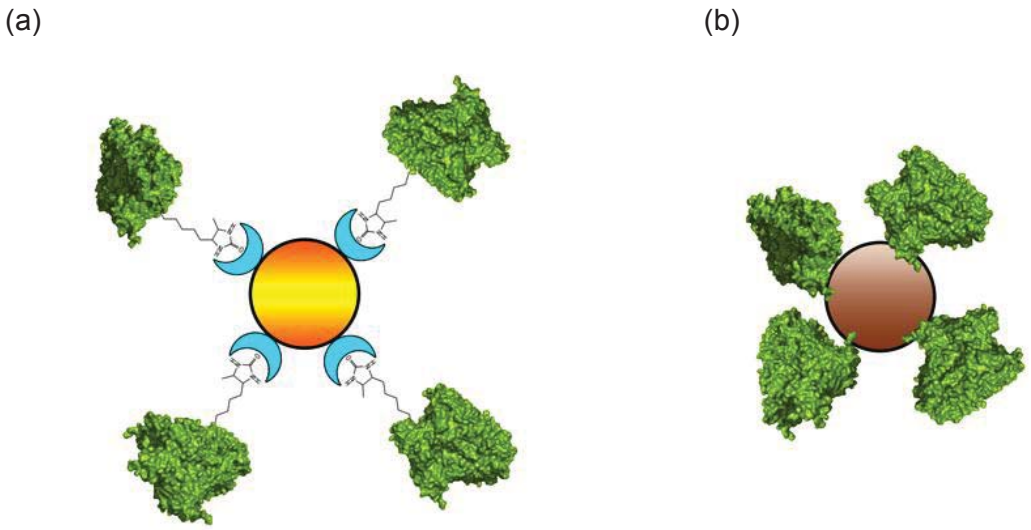

Figure 2. Two-step selection of DXS inhibitors. (a) Schematic representation of the streptavidin-coated beads used during rounds I and II of the first selection step to retain desthiobiotin-modified DXS and the phages that are bound to it (phages not shown). (b) Schematic representation of the nickel-beads used during round III of the first selection step and rounds I and II of the second selection step to retain DXS that has an N-terminal His-tag and the phages that are bound to it (phages not shown).

3.5 First phage display

As mentioned above, a commercially available M13 library PhD12 (NEB E8111L) was used for the first phage-display selection against *D. radiodurans* DXS. After three rounds of selection, the analysis of the sequences obtained clearly shows that a true selection occurred: we found several sequences to be repeated multiple times indicating their enrichment in the population (Table 2). However, there is no strong evidence of any consensus motif that would suggest a specific selection of binders targeting one particular region of *D. radiodurans* DXS. We took the four most prevalent sequences (**P1**–**P4**, Table 2) and tested the synthesized peptides for their inhibitory activity against *D. radiodurans* DXS.

The peptides were dissolved in DMSO (**P1** and **P2**) or water (**P3** and **P4**), and their inhibitory activities against *D. radiodurans* DXS were tested. To not miss out on potential slow binders, we investigated the influence of incubating the peptides with *D. radiodurans* DXS in Tris-HCl buffer (pH = 7.6) for 30 hours both at room temperature and at 4 °C.

Table 3. List of amino acid sequences obtained after the first phage display.

Sequence ID[a]	Sequence[b]	Peptide ID[c,d]	Sequence ID[a]	Sequence[b]	Peptide ID[c,d]
07AJ42	VNHEYKLHSIKY		07AJ68	ELQIGSWRMPPM	
07AJ43	TAELYPDLQSSQ	**P2** (x)	06DB70	SERLMTPPKLFR	
07AJ47	DDTYPSRPVYLK		07AJ71	MTHKQMHKHHGL	
07AJ52	DLYLSHGAPPQH		07AJ72	LVSLTPPWINVD	
07AJ53	HVTHNITNESNS		07AJ73	SSAQMNLNTFLN	**P13**
07AJ55	ARMTFSQMSPHT		06DB52	PVNKQHTSLQNN	**P1** (x2)
07AJ59	TGSIRPKLHASP		06DB54	LGSHNIRLGEGS	
07AJ60	MSSRSRPHINSL	**P3** (x3)	06DB58	YPHPIRQNFFAY	
07AJ61	QLARMSSLHVPM		06DB61	KSHTENSFTNVW	
07AJ63	EDARRPPTSTEH	**P4** (x4)	06DB62	KLPPMNSDSMVW	
07AJ64	SHEISRITAVSK		06DB68	HMNAHLTFQSAI	
07AJ67	VDMVTKQLLEYP		06DB69	DAVKTHHLKHHS	

[a] Sequencing file identification number.
[b] Peptide sequences were generated by translating the sequenced DNA considering the “amber mutation” codon usage i.e., the co-don TAG is translated with the amino acid Gln.
[c] Peptide IDs (**P1**, **P2**, **P3**, **P4**) are assigned to every sequence tested. [d] The values in brackets correspond to the times the se-quence has been found to be repeated.

We noticed that the peptides dissolved in DMSO gave better results when incubated at 4 °C, whereas peptides dissolved in water showed the maximum inhibition at room temperature. The best results were obtained for **P2** (50% of inhibition at 1000 μM after 30 hours incubation at 4 °C) and particularly **P3** (47% of inhibition at 250 μM, after 30 hours incubation at room temperature) (Table 4). Both **P2** and **P3** contain two adjacent Ser residues in the sequence: this Ser-Ser motif might function as a fingerprint for the recognition of the TDP-binding pocket of *D. radiodurans* DXS. Some preliminary modeling studies aimed at rationalizing the role of the Ser-Ser-(Ser) motif will be shown in Section 3.7.

Table 4. List of peptides selected from the first phage display and their inhibitory activities against *D. radiodurans* DXS, both in direct measurements and after incubation.

Peptide ID[c]	Solvent[d]	Direct measurement	Incubation[a]	
		IC_{50} (μM) [b]	% of inhibition r. t. [b]	% of inhibition 4 °C[b]
P1	DMSO	>1000	0% at 1000 μM	30% at 1000 μM
P2	DMSO	>1000	0% at 1000 μM	50% at 1000 μM
P3	H_2O	>250	47% at 250 μM	0% at 250 μM
P4	H_2O	>1000	30% at 1000 μM	30% at 1000 μM

[a] Peptides were incubated in Tris-HCl buffer (pH = 7.6) with *D. radiodurans* DXS for 30 hours at room temperature and/or 4 °C.
[b] IC_{50} values and percentage of inhibition were determined using a spectrophotometric assay. The values reported in the table correspond to the maximum concentration of the peptide, which was soluble in the assay conditions.
[c] **P1**–**P4** are amidated at the C-terminus.
[d] It refers to the solvent (H_2O or DMSO) used to prepare the stock solutions. When DMSO was used, the biochemical evaluation of IC_{50} was carried out according to the tolerance of DXS with respect to DMSO (up to 3%), determined as described in the Chapter 3.10.

3.6 Second phage display

We performed a second phage-display selection using a custom-made library taking into account the Ser-Ser motif. After the selection, the eluted phages were sequenced, and the results are shown in Table 5. The list contains some sequences that were present in the initial library, such as A10, A06, A01 and D02. Whereas A10 is a non-specific protein binder that we have found several times in other displays,[23] A06 is repeated several times and so it may be a DXS binder not discovered during the first display. The last two sequences (A01 and D02) might be contaminants given that they are not repeated.

Focusing on the sequences containing the Ser motif, we can see that some of them are repeated (*e.g.,* **P9**), whereas others are not repeated but contain some recurring motifs like the presence of additional Ser residues and multiple aromatic amino acids within the sequence (marked in bold and underlined in Table 5). Moreover, we observed that all the peptides contain at least one Pro residue, preferentially in the central part of the sequence, which might play a role in defining conformational preferences.

Table 5. Peptide sequences obtained after the second phage display.

Sequence ID	Sequence[a, b]	Peptide ID[c]	Sequence ID	Sequence[a, b]	Peptide ID[c]
A01	KAIRTRGKRPQY		B12	VSS**S**I**F**PIALPD	**P11**
A02	**Y**SSTI**Y**TPTAVG	**P5**	C02	HSSPVQTD**W**ITV	**P9** (x4)
A03	GSSLL**YS**G**S**GPA	**P6**	D02	THPSTKVPGTPA	
A06	MAIPTRGKMPQY	**P12** (x8)	E05	ASSVI**S**PR**W**LL**W**	
A10	ALWPPNLHAWVP[d]		E07	ALWPPNLHAWVP[d]	
A11	**S**SSPVA**W**ALAMR	**P7**	F09	TSSAAAP**YY**SPP	
B02	HSSPP**F**P**W**LLVT	**P10**	G05	VSSMKGPTL**S**TN	
B07	DSS**S**GL**Y**RPL**S**	**P8**	H06	DSST**W**L**F**L**SSY**R	

[a] Peptide sequences were generated by translating the sequenced DNA considering the "amber mutation" codon usage, i.e., the codon TAG is translated with the amino acid Gln [b] Additional Ser residues and aromatic residues are in bold and underlined. [c] The values in brackets correspond to the times the sequence has been found to be repeated. [d] Indicates a contaminant sequence, which non-specifically recognizes any protein.

We investigated a set of new peptides obtained from the second phage display, including the sequence 07AJ73 (**P13**) from the first round of phage display, considering that it contains a Ser-Ser motif at the N-terminus of the sequence. We tested the corresponding peptides (Table 6, **P5**–**P12**, **P13**) for their inhibitory activity against *D. radiodurans* DXS. Biochemical evaluation of **P7** resulted in an IC_{50} of 13 ± 3 μM, while the other peptides did not show any inhibition or very weak inhibitory activity (*e.g.*, **P10**, 20% inhibition at 1000 μM). As for the biochemical evaluation of the peptides originating from the first round of phage display, we included an incubation step not to miss out on potential slow binders, enabling us to confirm the inhibitory potency of **P7** (IC_{50} = 62 ± 13 μM after 30 hours incubation at 4 °C) and to identify **P13** as a double-digit micromolar inhibitor of D. *radiodurans* DXS (IC_{50} = 49 ± 11 μM). The accurate concentration of **P7** in solution was determined by UV spectrophotometry taking advantage of the fact that **P7** contains a single Trp residue. As a consequence, the IC_{50} dropped down to 9.5 ± 2 μM. Unfortunately, it was

not possible to determine the accurate concentration of **P13** in solution by absorbance measurements, due to the absence of Trp or Tyr residues in the amino acid sequence.

Table 6. List of peptides selected from the second phage display and their inhibitory activities against *D. radiodurans* DXS, both in direct measurements and after incubation.

Peptide ID[e]	Solvent[g]	Direct measurement	Incubation[a]	
		IC_{50} (μM) [b]	IC_{50} (μM) r. t.	IC_{50} (μM) 4 °C
P5	DMSO	>1000	-	>1000[c]
P6	DMSO	>1000	-	>1000[d]
P7	H_2O	13 ± 3 (9.5 ± 2)[f]	-	62 ± 13
P8	H_2O	>500	>1000	-
P9	H_2O	>500	>500	-
P10	DMSO	>1000[c]	-	>500
P11	DMSO	>1000	-	>1000
P12	H_2O	>1000	>1000	-
P13	DMSO	>50	-	49 ± 11

[a] Peptides were incubated in Tris-HCl buffer (pH = 7.6) with *D. radiodurans* DXS for 30 hours at room temperature and/or at 4 °C.
[b] IC_{50} values and percentage of inhibition were determined using a spectrophotometric assay. The values reported in the table correspond to the maximum concentration of the peptide, which was soluble in the assay conditions.
[c] 20% inhibition at 1000 μM.
[d] 40% inhibition at 1000 μM.
[e] **P5**–**P13** are not amidated at the C-terminus.
[f] IC_{50} value determined based on the accurate concentration of **P7** in the stock solution, as determined by absorbance measurements.
[g] It refers to the solvent (H_2O or DMSO) used to prepare the stock solutions.

Comparing the sequences of the two most successful peptides, **P7** (containing a Ser-Ser-Ser motif) and **P13** (containing a Ser-Ser motif), it is clear that the Ser residues at the N-terminus are essential for their inhibitory potency. It is important that the sequence starts with the Ser motif. In fact, peptides bearing a different amino acid at the N-terminus, right before the Ser motif, (*e.g.*, **P5**, **P6**, **P8**), display no inhibition or very weak inhibition of *D. radiodurans* DXS. The use of a coupled spectrophotometric assay, requires a follow-up assay with the auxiliary enzyme, IspC. Therefore we tested **P7** for its inhibitory potency against *E. coli* IspC and found that it has an IC_{50} value of 398 ± 61 μM. The fact that **P7** acts both as an inhibitor of DXS and of IspC, can be considered an advantage. In fact, the possibility of targeting multiple enzymes of the MEP pathway with one compound looks

very appealing for the development of novel drugs, potentially able to overcome drug-resistance.[24]

3.7 Rationalization of the binding mode – Ala scan

To elucidate the contribution of each amino acid residue, we performed an Ala scan of **P7**, which displays low micromolar activity against *D. radiodurans* DXS also without pre-incubation. The nine peptides corresponding to the systematic replacement of non-Ala residues with Ala, (**P7b**–**l**, Table 7) were synthesized and tested *in vitro* against *D. radiodurans* DXS without pre-incubation. To verify the influence of the free C-terminus, we also tested the C-amidated derivative of **P7** (**P7a**, Table 7). Given that some derivatives of **P7** were insoluble in water even at very low concentration, we decided to use them as stock solutions in DMSO. To start with, we tested the reference peptide **P7** in DMSO so as to have a reference value for its inhibitory activity in both solvents. We found its IC_{50} against *D. radiodurans* DXS to be higher (IC_{50} = 93 ± 17 μM) than when tested as a stock solution in water (IC_{50} = 13 ± 3 μM). Replacement of one of the three Ser residues at the N-terminus of **P7** (derivatives **P7b**–**d**, Table 5) resulted in a significant loss in the inhibitory activity of the corresponding peptides (IC_{50} (**P7b**–**d**) > 500 μM). This result suggests that the Ser-Ser-Ser motif at the N-terminus of the peptide chain plays an essential role in the protein-recognition process and thus in the inhibitory activity observed for **P7**. When replacing the Pro residue at the fourth position of the chain with an Ala residue, the inhibitory activity is improved with respect to **P7** (**P7e**, IC_{50} = 43 ± 13 μM). This fact might indicate that the presence of a small, hydrophobic residue, is the most suitable for this position, but the specific conformational preferences imposed by Pro might disfavor the interaction between **P7** and *D. radiodurans* DXS. In general, replacement of the hydrophobic core of the peptide, up to the C-terminus (**P7f**–**i**) leads to at least a ten-fold loss in inhibitory activity. Even the presence of Ala instead of Val or Leu (**P7f** and **P7h**, respectively) causes substantial or complete loss of activity (**P7f**: IC_{50} > 300 μM; **P7h**: IC_{50} = 252 ± 23 μM), showing how important inter- or intramolecular hydrophobic interactions seem to be for the inhibitory activity of **P7**. Modification of the first residue at the C-terminus shows that there is room for improvement of the inhibitory potency of **P7**: the presence of an Ala rather than an Arg residue (**P7l**), doubled the inhibitory potency (IC_{50} = 47 ± 13 μM), suggesting that different, small, hydrophobic residues might be suitable in this position when optimizing the inhibitory activity of **P7**. On the other hand, amidation of the C-terminus (**P7a**) resulted in a complete loss of the inhibitory potency (IC_{50} > 300 μM).

Table 7. Ala scan of **P7**.

Peptide ID	Sequence	Solvent[a]	IC_{50} (μM)
P7 (ref)	SSSPVAWALAMR	H_2O	13 ± 3
P7 (ref)	SSSPVAWALAMR	DMSO	93 ± 17
P7a	SSSPVAWALAMR-NH_2	H_2O	>300
P7b	**A**SSPVAWALAMR	DMSO	>500
P7c	S**A**SPVAWALAMR	DMSO	>500
P7d	SS**A**PVAWALAMR	DMSO	>500
P7e	SSS**A**VAWALAMR	DMSO	43 ± 13
P7f	SSSP**A**AWALAMR	H_2O	>300
P7g	SSSPVA**A**ALAMR	DMSO	422 ± 99
P7h	SSSPVAWA**A**AMR	H_2O	252 ± 23
P7i	SSSPVAWALA**A**R	H_2O	170 ± 23
P7l	SSSPVAWALAM**A**	DMSO	47 ± 13

[a] It refers to the solvent (H_2O or DMSO) used to prepare the stock solutions.

On the way to rationalize the importance of the Ser-Ser-Ser motif, we did preliminary modeling studies with the software Moloc, in which we performed an energy minimization of this trimer both within the TDP- and within the substrate-binding pockets of *D. radiodurans* DXS (Figure 3a and 3b, respectively). As one can observe, both pockets are likely to be occupied by the trimer, which is engaged in numerous hydrogen-bonding interactions with the protein. In particular, in the substrate-binding pocket, the trimer is able to interact with the residues, which are supposed to be involved in the catalytic process of DXS or in the substrate-recognition event (Figure 3b).

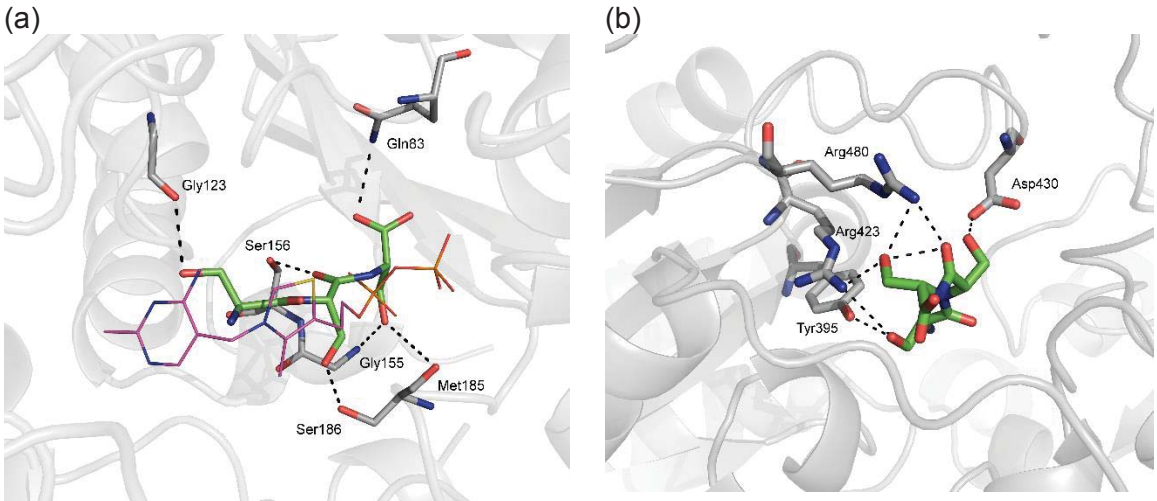

Figure 3. Modeled binding mode of the trimer Ser-Ser-Ser in: (a) the TDP-binding pocket and (b) the substrate-binding pocket of *D. radiodurans* DXS (PDB: 2O1X). Color code: protein (shown as cartoon): C: gray; O: red; N: blue; S: yellow. Ser-Ser-Ser skeleton: C: green. TDP (shown as lines): C: purple. Hydrogen bonds below 3.5 Å shown as dashed lines.

3.8 *In vitro* activity against *Mycobacterium tuberculosis* DXS and cell-based assays

We tested our two most promising peptides, **P7** and **P13** also against *M. tuberculosis* DXS but they did not show any inhibitory potency in this case. As discussed already in Chapter 2 and as we will discuss further in the coming chapters, despite having a high degree of sequence and presumably also structural homology, surprisingly most often our inhibitors do not show the same behavior against the two orthologues of DXS.
P7 was tested also in cell-based assays against multi-drug resistant (MDR) strains of *M. tuberculosis*, namely 1011200345 (resistant to isoniazide, rifampicine, pyrazinamide, ethambutol, streptomycin, ciprofloxacin and clarithromycin), 1010901458 (resistant to isoniazide, rifampicine, ethambutol and streptomycin) and 101100966 (resistant to isoniazide, rifampicine, ethambutol, streptomycin and pyrazinamide). In all cases, the Minimum Inhibitory Concentration (MIC) value turned out to be > 20 μM. The fact that no cell-based activity was observed, does not come as a surprise. In fact, standard peptide sequences composed of natural amino acids are, besides few exceptions, too polar to be able to cross cellular membranes and have to be carefully optimized for their physicochemical properties and pharmacokinetic profiles. Moreover, *M. tuberculosis* is known to have a particularly thick and hydrophobic cell wall,[25] which renders the optimization of any peptidic inhibitor, a necessary step to develop any hit compound into an efficient antituberculotic agent.

3.9 Conclusions and outlook

In summary, we reported the identification of the first peptidic inhibitor of the enzyme DXS. In fact, all reported inhibitors of DXS are small, organic molecules. The use of phage display enabled us to rapidly and efficiently identify peptidic inhibitors with an activity in the low micromolar range (**P7**), comparable in potency to the most active small-molecule inhibitors discovered so far (Chapter 1, Figure 11). The Ala scan of **P7** demonstrated the essentiality of the N-terminal Ser-Ser-Ser motif in the amino acid sequence, together with other key parts of the sequence that we will exploit to enhance the inhibitory potency of **P7** in future optimization cycles. Therefore, our work sets the stage for the development of potent, peptidic inhibitors of DXS as anti-infective agents.

Truncation studies to understand which amino acid residues are essential for the binding – and therefore for the inhibitory potency – of **P7** to DXS, should be carried out. Certainly, the trimer consisting of Ser-Ser-Ser should be tested.

One should also exploit the advantage of phage display, which can generate wide libraries of peptides without the need for their costly and time-consuming synthesis and allows to rapidly screen them as binders – and therefore potential inhibitors – of a certain target. For example, a further phage display cycle, could include the screening of a library containing only two randomized positions, namely the Pro and the Arg residue (SSS***X***VAWALAM***X***), which did not lead to any drop of the inhibitory potency when replaced by Ala. This could help to identify suitable amino acid residues for these two positions, enhancing the inhibitory potency of **P7** even more.

3.10 Experimental

Bacterial strain. *E. coli* ER2738 (NEB E4104S) (Geno-type: F´*proA+B+ lacIq Δ(lacZ)M15 zzf::Tn10(TetR)/ fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5*) is a male *E. coli* in which the F´can be selected for using tetracycline, it allows Blue/White screening and it is an amber mutant strain. It was used for cloning and expression of the phage library, for titering and for the inoculation of the sequencing plates.

Phage Display I. The first phage-display selection protocol was carried out using the commercially available M13 library PhD12 (NEB E8111L) consisting of M13 phages expressing a 12aa peptide at the N-terminus of each coat protein pIII a small linker Gly-Gly-Gly-Ser was inserted between the peptide and the coat protein to increase the conformational freedom of the exposed peptides and to minimize the contribution of the protein pIII to the overall binding. The schematic sequence of this library is N-term-X_{12}-GGGS-p3-C-term. Three rounds of selection were performed in PBS buffer (1 mL, sodium phosphate 50 mM, NaCl 150 mM, pH 7.5) incubating 10E10 phages with *D. radiodurans* DXS (1 mg) in a 2 mL protein-low-binding tube for 30 min on ice. For the first two rounds, DXS functionalized with NHS-Desthiobiotin and Dynabeads MyOne Streptavidin C1 (Invitrogen 65001) were used to capture DXS from the solution. For the third round, to avoid selection of phages against streptavidin and given that *D. radiodurans* DXS contains an N-terminal His-tag, MagneHis Ni-Particles (Promega V8560) were used as capturing system. After the incubation step, 0.1 mL of beads were added to the solution, which was mixed in a thermo shaker at 4 °C for 15 min. The tube was placed in a magnetic rack to allow for the adhesion of the magnetic beads on one side of the tube. Phages expressing

DXS-binders were retained on the bead surface, the buffer containing unbound phages was gently discarded from the tube. To further remove weakly bound phages, the beads were washed with PBST (10 x 1 mL PBS with 0.05% Tween 20) whilst retaining them using a magnetic rack. The elution of strongly bound phages from the beads was achieved by suspending the beads in the elution buffer (1 mL, 1 mM Biotin in PBS for rounds 1 and 2 and 500 mM imidazole in PBS for round 3). After separation of the beads, the solution containing the eluted phages was used to amplify the selected pool of phages by infecting a fresh culture of *E.coli* ER2738. Infection, production and purification of the phages were carried out following the manufacturer's manual. Peptides **P1**−**P4** were purchased from CASLO (Lyngby, Denmark) with purity > 97% according to HPLC.

Library design and cloning. A custom-made library was designed in order to include the motif Ser-Ser at the N-terminus of the peptide library. Two oligomers, one coding for the library itself and one used for cloning purposes were designed:

Library Oligo:

CATGTTTCGGCCGA$(MNN)_9$GGAGGAMNNAGAGTGAGAATAGAAAGGTACCCGGG

Extension Primer: CATGCCCGGGTACCTTTCTATTCTC

The "Library Primer" codes for the reverse strand of the library and it includes two flanking regions that contain the restriction site for KpnI/Acc651 and EagI needed for cloning into the M13KE vector. The random part of the peptide sequence is coded by NNK codons (reverse complement of MNN) where N is any of the bases while K represents G or T (thus, M represents C or A). An NNK codon can encode for all 20 amino acids but only for one stop-codon: TAG. Combining the use of NNK codons with amber mutant strains like *E. coli* ER2738, ensures that the whole library will code for full-length peptides. The "Extension Primer" is partially complementary with the library-coding oligomer and it was used to generate the dsDNA needed for the cloning. The preparations of the library duplex and the cloning were performed as indicated in the manufacturer's manual (NEB E8mL).

Phage Display II. Two rounds of selection were performed using a custom-made phage library. The schematic sequence of the expressed library is: N-term-XSSX9-GGGS-p3-C-term. TBS was used as incubation buffer, TBST (TBS with 0.05% Tween 20) as washing buffer, MagneHis Ni-Particles (Promega V8560) were employed for protein recovery and 1 mM TDP in TBS as elution buffer. TDP was chosen as competitive eluent to specifically elute peptides interacting with the TDP-binding site of DXS. The same procedure as described for Phage Display I was used with the following modifications: DXS (1 mg) was incubated simultaneously with phages from the new library, phages from the original PhD-

12 library were added to increase sequence complexity, and wild-type phages were supplemented to screen for non-specific binders. The different phage pools were mixed at a 1:1:1 ratio prior to incubation with the target.

Sequencing. The last elution fractions from the phage display experiments were serially diluted (1:10) and used to infect a fresh culture of *E. coli* ER2738. The infected culture was plated on LB-agar supplemented with Tetracycline, IPTG and XGal. Blue colonies resulting from phage infection were picked and sent for Sanger sequencing (at GATC Biotech) using a custom designed M13-specific sequencing primer (GTACAAACTACAACGCCTGT). Peptides **P5**–**P13** and **P7a**–**P7l** were purchased from ProteoGenix SAS (Schiltigheim, France) with purity > 95% according to HPLC.

Gene expression and protein purification of *D. radiodurans* and *M. tuberculosis* DXS. Gene expression and protein purification of *D. radiodurans* and *M. tuberculosis* DXS were performed as reported in Chapter 2.

Spectrophotometric assay for the determination of IC_{50} values against *D. radiodurans* and *M. tuberculosis* DXS. Direct measurements of the inhibitory activities with the spectrophotometric assay, were performed as reported in Chapter 2. The tolerance of DXS with respect to DMSO concentration was determined by measurement of the reaction velocity in the presence of different concentrations of DMSO. The activity of the enzyme was found to be stable in presence of up to 3% DMSO. For determining the inhibitory activity of the peptides after incubation, several solutions were prepared containing degassed Tris-HCl (pH = 7.6, 100 mM. 300 µL), *D. radiodurans* DXS (0.79 µM) and different concentrations of each peptide, with a dilution factor of 1:2 starting, when possible, from 1000 µM. The solutions were incubated at room temperature or at 4 °C for 30 hours. Preliminary control experiments have shown that the activity of *D. radiodurans* DXS is unchanged at room temperature or at 4 °C after 30 hours. After the incubation time, each incubated solution (95 µL) was transferred to a 96-well plate, and a buffer containing Tris-HCl (pH = 7.6, 100 mM) and D-glyceraldehyde-3-phosphate (4.0 mM) was added (47.5 µL). The reaction was started by addition of a buffer solution (47.5 µL) containing Tris-HCl (pH = 7.6, 100 mM), $MnCl_2$ (16 mM), dithiothreitol (DTT, 20 mM), NADPH (2.0 mM), sodium pyruvate (2.0 mM), TDP (4.89 µM) and *E. coli* IspC (8.2 µM). A control experiment with the enzyme incubated in Tris-HCl buffer (and DMSO when testing peptides as stock solutions in DMSO) at the same temperature and for the same time, was carried out in parallel to monitor for potential loss in activity of the enzyme itself, which has never been observed.

Determination of the exact concentration of P7 in solution by absorbance measurement. The concentration of **P7** in aqueous solution was calculated applying the

following equation: [Peptide concentration] mg/mL = (A280 x DF x MW) / ε, where A280 is the absorbance of the peptide solution at 280 nm in a 1 cm cell, DF is the dilution factor, MW is the molecular weight of the peptide and ε is the molar extinction coefficient of Trp at 280 nm (5690 $M^{-1}cm^{-1}$).

Gene expression, purification of *E. coli* IspC and biochemical evaluation of inhibitory activity against *E. coli* IspC by spectrophotometric assay. Gene expression and purification of *E. coli* IspC and biochemical evaluation of inhibitory activity of **P7** against *E. coli* IspC was performed as reported previsouly.[26]

Cell-based assays. A total of three MDR *M. tuberculosis* isolates, and one control strain (H37Rv) were selected and subcultured on a Middlebrook 7H10 tube until use. Susceptibility testing using the absolute concentration method were carried out by preparing 25-well plates with solid 7H10 medium containing different concentrations (20, 10, 5, 2.5, 1.25 μM) of **P7**. The plates were subsequently inoculated by adding 10 μL *Mycobacterium* suspension to each well. Then the plates were incubated at 35.5 °C in a CO_2 incubator. After appropriate incubation, the MICs were assessed. The reading of the plates was carried out when the bacterial growth on the two control wells without **P7** was sufficient, *i.e.,* when colonies were clearly visible.

3.11 References

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